Kidney International, Vol. 54 (1998), pp. 224–235

Effect of alternative peritoneal dialysis solutions on cell viability, apoptosis/necrosis and cytokine expression in human monocytes[1]

JOERG PLUM, MOHAMMAD REZA LORDNEJAD, and BERND GRABENSEE

Department of Nephrology and Rheumatology, Heinrich-Heine-University, Duesseldorf, Germany

Effect of alternative peritoneal dialysis solutions on cell viability, apoptosis/necrosis and cytokine expression in human monocytes.

Background. Cellular function, cell viability and the cytokine network of human monocytes are influenced by the specific composition of peritoneal dialysis (PD) fluids. In an *in vitro* study using isolated human blood monocytes, we investigated the effect of peritoneal dialysates containing amino acids (Amino) or glucose polymer (Glu-poly) instead of glucose (Glu) as the osmotic agent, and bicarbonate (Bic) or PBS instead of lactate (Lac) as a buffer.

Methods. The following parameters were studied: mitochondrial dehydrogenase activity (using the MTT assay), interleukin (IL)-6 and IL-8 release (ELISA) and cellular IL-6 mRNA expression after lipopolysaccharide (LPS) stimulation (using RT-PCR). FACS flow cytometry with annexin V and propidium iodide as markers and fluorescence microscopic methods were used to study the effects of the test fluids on cell necrosis and apoptosis.

Results. Glu/Lac pH 5.5 and Glu-poly/PBS pH 7.4 both significantly reduced mitochondrial dehydrogenase activity by more than 50% after 60 minutes of incubation (30.5 ± 7.6%, 42.5 ± 6.5%, referred to RPMI 1640 as 100%). Amino/Bic and Glu/Bic were both superior (MTT assay > 63%). The rate of necrotic cells after 15 minutes of incubation measured by FACS was mostly increased with Glu/Lac pH 5.5 (29.9 ± 4.0%). The rate of apoptotic cells, however, was not significantly different between the test solutions. The concentration of IL-6 in the supernatant of stimulated monocytes was highest with Glu/Bic (1023 ± 278 pg/ml) and Amino/Bic (776 ± 296 pg/ml) and lowest with Glu/Lac pH 5.5 (46 ± 22 pg/ml) and Glu-poly/PBS (32 ± 13 pg/ml). IL-8 release from stimulated monocytes showed a similar pattern. Glu-poly/PBS showed a suppressive effect on IL-6 mRNA expression (ratio IL-6/β-Actin, 0.4 ± 0.25 vs. RPMI 1.5 ± 3.6).

Conclusions. Bicarbonate buffered solutions both with glucose or amino acids as osmotic agents were superior when regarding cell metabolism, viability and cytokine release, while lactate buffered solutions and Glu-poly/PBS showed some reduced biocompatibility pattern for monocytes *in vitro.*

Continuous ambulatory peritoneal dialysis (CAPD) as a renal replacement therapy has been established as a safe and widely applied method. Despite continuous improvement of the technical equipment, peritonitis is still an important problem and the major reason for system failure. Peritonitis is associated with an increase in peritoneal permeability [1, 2], loss of ultrafiltration und in some cases sclerosis of the peritoneum [3]. Therefore, with regard to short-term and long-term outcome it remains an important issue to further reduce the rate of peritonitis and to improve cellular biocompatibility of the dialysate.

The local host defense mechanisms within the peritoneal cavity play a pivotal role for the prevention of peritoneal infection. Since the normal peritoneal system in healthy subjects can overcome some bacterial contamination (for example, during surgery) without developing peritonitis, the peritoneal dialysis (PD) patient is highly susceptible to minor system contamination [4]. Negative effects of PD fluids on cellular host defense of monocytes are well known. Lactate as a buffer substance, a low pH value and high osmolality impair cellular function and viability (such as, measured by phagocytosis activity, cellular ATP content or LDH release) [5–7]. The same can be found for the release of inflammatory interleukins within the cytokine network [5, 7]. In the peritoneal cavity an equilibrium between the fresh dialysate and the blood compartment is reached via diffusion between 15 and 30 minutes (concerning pH) and several hours (concerning osmotic agents and peptides/proteins from the serum). However, the early phase when undiluted dialysate is instilled into the peritoneum seems to be most harmful [6].

Interleukin (IL)-6 and IL-8 are important mediators of the inflammatory response. Besides mesothelial cells, macrophages represent the major source of IL-6 [8] within the peritoneal cavity. Interperitoneal IL-6 might have a modulating effect on the inflammatory cascade and has a potential permeablitiy increasing effect on the peritoneum [9]. IL-8 is a chemotactic cytokine with proinflammatory and growth-promoting activities [10]. It is released by a variety of cell types, mainly by monocytes/macrophages [11]. IL-8 exhibits potent chemotactic activity for neutrophils [12], but it is also a chemoattractant factor for T-lymphocytes [13] and basophils [14]. Both cytokines are important promoters of cellular host defence.

[1] **See *Editorial* by Cohen, p 283**

Key words: dialysate, amino acids, glucose polymer, lactate buffer, bicarbonate, osmotic agents, dehydrogenase activity.

Received for publication August 5, 1997
and in revised form January 15, 1998
Accepted for publication January 27, 1998

Table 1. Composition of the control media and test solutions

Composition	Reference media		Test solutions					
	RPMI 1640	PBS	Amino/Bic	Amino/PBS	Glu/Bic	Glu/Lac pH 5.5	Glu/Lac pH 7.5	Glu-poly/ PBS
Amino acid *g/liter*	1		10[a]	10[a]				
Glucose *g/liter*	2				13.6	13.6	13.6	
Lactate *mmol/liter*						40	40	
Bicarbonate *mmol/liter*	23.8		34		34			
Anorg. phosphate *mmol/liter*	5.6	11.3		9.9				8.8
Glucose polymer (molecular wt 15000) *g/liter*								75
Sodium *mmol/liter*	138	146	134	154	134	134	137	148
Calcium *mmol/liter*	0.9		1.25		1.25	1.25	1.25	
Magnesium *mmol/liter*	0.4		0.5		0.5	0.25	0.25	
Chloride *mmol/liter*	108	139	103	143	103	104	104	137
Osmolality *mosm/kg*	290	290	369	364	361	358	360	318
pH value	7.5	7.4	7.5	7.5	7.5	5.5	7.5	7.4

Abbreviations are in the Appendix.
[a] Composition of 70% essential amino acids and 30% nonessential amino acids

While conventional markers such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, adenosine triphosphate (ATP) assay and lactate dehydrogenase (LDH) release have been studied as global markers for cell viability, little is known about the influence of dialysates on apoptosis. Most cells from higher Eukaryotae have the ability for self-destruction by activation of an intrinsic cellular suicide program when they are no longer needed or have become seriously damaged. This normal physiological process is referred to as programmed cell death (apoptosis) [15]. Apoptosis is characterized by chromatin condensation, membrane blebbing, nuclear and cytoplasmatic shrinkage, and the generation of DNA fragments through the activation of endogenous endonuclease without generating an inflammatory response. This is in contrast to the type of cell death known as necrosis, which is characterized by cell lysis and activation of a local inflammatory response [15–19].

Little is known thus far about the influence of amino acids or polyglucose as osmotic agents or different buffers on the mentioned parameters. Amino acids and polyglucose have gained increasing clinical interest for indications as malnutrition or loss of ultrafiltration. Bicarbonate has proven to be a superior buffer in combination with glucose with regard to biocompatibility, but the effects in combination with amino acids are still open. The current *in vitro* study aimed at characterizing the effects of alternative osmotic agents and buffers on monocyte function.

METHODS

COMPOSITION OF TEST SOLUTIONS AND CONTROL MEDIUM RPMI 1640

Six test solutions were investigated: glucose/lactate (Glu/Lac) pH 5.5 and pH 7.5; glucose/bicarbonate (Glu/Bic) pH 7.5; amino acid/bicarbonate (Amino/Bic) pH 7.5; amino acid/PBS buffer (Amino/PBS) pH 7.5; glucose polymer/PBS-buffer (Glu-poly/PBS) pH 7.4; and RPMI 1640, PBS buffer as an internal control media (Table 1). PBS buffer pH 7.4 proved to be a simple medium with good biocompatibility patterns according to the parameters tested. It was therefore used as an internal reference in addition to RPMI 1640. Amino acids and Glu-poly were combined with this medium to focus on the specific effects of the osmotic agent when combining with a clearly superior buffer system (though not suited in this form for clinical application).

Glucose, glucose polymer (dextrin), amino acids and L-lactic acid were supplied with high purity from Sigma (Deisenhofen, Germany). An 8.4% sodium hydrogen bicarbonate solution and PBS were supplied from Pharma Halmen GmbH (Halmen, Germany). All test solutions were sterile filtered with a 0.45 μm filter and subsequently with a 0.2 μm pyrogen-free filter (Satorius AG, Göttingen, Germany).

Endotoxin-assay

The endotoxin concentration in all solution was measured with the Limulus Amebocyte Lysate assay, QCL-100 (Bio-Whittaker, Walkersville, USA; Boehringer Ingelheim, Germany). In all PD test solutions and RPMI the endotoxin level was determined to be low ($\leq$2 U/liter).

Cells and culture conditions

Human blood monocytes were obtained from healthy donors (buffy coats). Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque (Pharmacia Biotech, Sweden), according to Böyum [20]. Two $\times$ 10^6/ml cells in 50 ml RPMI 1640 medium (Biochrom, Germany) with supplements [10% fetal calf serum (FCS; Gibco BRL, Scotland, UK), 2 mM L-glutamine (Gibco BRL), 0.05 IU/ml penicillin and 0.05 μg/ml streptomycin (ICN, USA)] were cultured in 175 cm^2 tissue culture flasks (Becton Dickinson,

NJ, USA) in an atmosphere of 5% CO_2 at 37°C for two hours. Lymphocytes were removed by washing and monocytes were detached by gently shaking at 4°C. A total of 90.1 ± 4.4% of the cells were determined to be viable by the trypan blue exclusion test; 93% ± 4.4% of the isolated cells were monocytes, according to peroxidase staining using benzidine dihydrochloride (Sigma, Deisenhofen, Germany).

Incubation procedure

One milliliter of cell suspension (8 to 10 × 10^6 monocytes) in PBS was incubated with 25 ml of the test fluids (Table 1) for 15 minutes at 37°C, 5% CO_2 in 75 cm^2 tissue culture flasks and stimulated with 10 μg/ml LPS (*E. coli* serotype 055/B5; Sigma, Deisenhofen, Germany). The test fluids were warm and pCO_2 equilibrated (after standing in the incubator) when the pH was adjusted. PBS and RPMI (without supplements or FCS) were used as reference media for the fresh dialysates. After 15 minutes of incubation with the test fluids (except for the MTT assay, where intervals between 5 to 60 min were evaluated) 26 ml RPMI 1640 and 20% FCS was added, and incubation was continued for 16 hours (recovery period). All media had a physiological pH value and an osmolality between 290 and 369 mOsm/kg after addition of RPMI. The pH of the media after incubation in the CO_2 atmosphere was controlled and did not significantly decrease with time (<0.3 pH units after 16 hr). The same incubation procedure was applied for the MTT assay, apoptosis/necrosis assay and cytokine measurement.

MTT assay

The assay relies on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by succinate-dehydrogenase activity in active mitochonodria [21]. Blood monocytes were incubated for 5 to 60 minutes in the undiluted test fluids (or RPMI as the reference) followed by the incubation scheme as described above. After 16 hours recovery in RPMI plus supplements 1 × 10^6 monocytes were incubated with the MTT solution for further four hours at 37°C, 5% CO_2. Ten percent SDS was then added and the culture plates were incubated overnight. Formazan crystals were solubilized and the generation was quantified using a spectrophotometer [22]. The extinction coefficient of RPMI 1640 was referred to as 100%. The data from monocytes submitted to a freeze/thaw cycle and incubated with RPMI served as a negative control.

Apoptosis and necrosis assays

FACS. Cell staining was performed with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI) [18, 19]. Early stages of apoptosis (without loss of cell membrane integrity) are characterizied by the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer [18]. Annexin V is a Ca^{2+} dependent phospholipid-binding protein with high affinity for PS [19]. After the incubation procedure described above monocytes were washed with an ice-cold electrolyte solution (Pharmacia, Erlangen, Germany) and resuspended in cold diluted binding buffer (Hölzel Diagnostika, Köln, Germany) at a concentration of 10^6 cells/ml. A total of 440 μl of the cell suspension was incubated with 10 μl of the FITC-labeled annexin V (final concentration 2 μg/ml; Hölzel Diagnostika) and 50 μl propidium iodide (final concentration 1 μg/ml) for 10 minutes in the dark at 4°C. The cells were analyzed by FACScan flowcytometry (FACSort; Becton Dickinson, USA). The gate selected corresponded to monocytes according to the dot-blot histogram, and 10,000 to 50,000 blood monocytes were counted. The differentiation of cellular distribution was received by a contour line representation. The inter- and intra-assay variation coefficient was < 6% ($N = 5$). RPMI served as the reference medium. A positive control for the induction of apoptosis was introduced by a murine monoclonal anti-Apo-1/Fas IgG antibody (Hölzel Diagnostika) at a concentration of 1 μg/ml added to Ringer's solution for two hours at 4°C.

AO staining, TUNEL assay. The validity of the results concerning the incidence of apotosis and necrosis was controlled for by investigating an aliquot of the cells by fluorescence and light microscopy. Two × 10^6 cells were incubated on ice with acridin orange (Sigma) for 15 minutes at a concentration of 10 μg/ml [23]. The cellular shape and typical color was judged under UV light according to nuclear condensation/fragmentation and cytoplasma condensation. The ratio of cells with these changes was evaluated semiquantitatively.

Additionally enzymatic *in situ* labeling of apoptosis-induced DNA strand breaks was carried out by using a kit based on the TdT-mediated dUTP nick end labeling (TUNEL) method (*In Situ* Cell Death Detection Kit, Fluorescin, #1684795; Boehringer Mannheim, Germany). The test was performed after the usual recovery period of 16 hours. TUNEL positive nuclei were observed and counted in fluorescent micrographs. A positive control was introduced by treating permabilized cells with micrococcal DNase I (10 μg/ml, 10 min).

Cell membrane disruption as a sign of necrosis (or occurring in the late phase of apotosis, that is, secondary necrosis) was tested by vital staining with trypan blue.

The reliability of the FACS results was controlled for by these microscopic methods.

Cytokines

The concentration of IL-6 and IL-8 was determined in the supernatants of monocytes (from buffy coats) after 16 hours of incubation (Human IL-6 and IL-8, DuoSeT™; Genzyme, USA). Cells were stimulated with 10 μg/ml LPS. Unstimulated monocytes in RPMI served as a negative control. Response curves for LPS-induced cytokine release

were established during the initial time-course experiments with incubation periods between 0 hours and 48 hours, and showed that a 16 hour incubation/stimulation was well suited for the measurement of mRNA expression and cytokine release. Activated macrophages isolated from the effluent of three patients with peritonitis (staphylococcus peritonitis, cells isolated after a six hour dwell before the onset of antibiotic treatment) served as a positive control for the adequacy of LPS stimulation. The lower detection limit of the ELISAs was 0.156 pg/ml and 15.6 pg/ml, respectively. The specifity of all assays inculding potential martix effects (dialysates) was tested for by sample spiking and dilution. Interference between the test solutions and the ELISA system was excluded and accuracy of the assay was controlled. No interference with the test solutions could be found. Inter- and intra-assay variation coefficient was $< 10\%$ ($N = 10$).

RNA isolation

Total RNA was extracted from cultured cells by the Trizol™ reagent (Gibco BRL). RNA isolation was performed according to Chomczynski and Sacchi [24]. RNA was quantified by measuring optical density at 260 and 280 nm. Total RNA obtained was 10 to 15 μg/5 to 10×10^6 cells.

Reverse transcription and polymerase chain reaction amplification

For the RT-PCR assay, 1.5 μg total RNA was reverse transcribed into cDNA by using first-strand cDNA Synthesis Kit (Pharmacia Biotech, Freiburg, Germany). The tubes were incubated at 37°C for one hour and the reverse transcriptase was inactivated by a 10 minutes incubation at 70°C. Ten microliters of reverse transcribed RNA (100 ng cDNA) was amplified by PCR in a total volume of 50 μl using 1 U *Taq*-Polymerase (Pharmacia Biotech, Freiburg, Germany), 100 μM dNTPs (Boehringer Mannheim) and 25 pmol of each primer in PCR buffer. Sense primers for β-actin were [25] 5′-AGA GAT GGC CAC GGC TGC TT-3′, and antisense primers were 5′-ATT TGC GGT GGA CGA TGG AG-3′, which yielded a 406 base-pair PCR product. Sense primers for GAPDH were 5′-ACA GTG CAC TCT GGA CAG T-3′, and antisense primers were 5′-TCC AGC TGC AGT GTG TCC A-3′, which yielded a 265 base-pair PCR product. Sense primers for IL-6 were [26] 5′-GCT ACA TTT GCC GAA GAG CC-3′, and antisense primers were 5′-TAC ATC CCT CGA CGG ATC TC-3′, which yielded a 456 base-pair PCR product. cDNA was amplified in a thermal cycler (PTC-100™; Biozym, USA). PCR conditions were established by RNA (cDNA), primer and enzyme (*Taq*-DNA-polymerase) titration, before the amplification reactions. Twenty microliters of each reaction PCR product were loaded onto a 2% agarose gel containing 0.5 μg/ml ethidium bromide. The signal intensity of the PCR products was quantified by UV-Light with a CCD-Camera (Herolab, Wiesloch, Germany). The IL-6 specific mRNA level was calculated by the ratios of IL-6 and β-actin or GAPDH as endogenous standards. mRNA expression in unstimulated monocytes (RPMI, without LPS) and activated macrophages from the peritoneal effluent of patients with peritonitis served as control.

Data analysis

All data are expressed as mean ± SD. Statistical significance was calculated using the Student's *t*-test, *P* values less than 0.05 were considered to be significant. For multiple comparisons Bonferroni correction was applied.

RESULTS

Time dependent effects of dialysis fluids on cell viablity

With the progression of incubation time (5, 15, 30, 45, 60 min), the mitochondrial dehydrogenase activity (MTT test) decreased in a different degree for the dialysis fluids tested (Fig. 1). Glu/Bic pH 7.5, Amino/Bic pH 7.5 and Amino/PBS pH 7.5 showed the highest mitochondrial dehydrogenase activity, whereas Glu/Lac pH 7.5, Glu-poly/PBS pH 7.4 and Glu/Lac pH 5.5 showed the lowest mitochondrial dehydrogenase activity at all incubation times. Even after five minutes of incubation, the MTT test activity was reduced to 62 ± 14% with Glu/Lac pH 7.5 ($P < 0.05$, vs. RPMI) and to 52 ± 7% with Glu/Lac pH 5.5 ($P < 0.001$, vs. RPMI). A low activity was also measured for Glu-poly/PBS pH 7.4 with 55 ± 8% ($P < 0.001$, vs. RPMI).

Mitochondrial dehydrogenase activity after 45 minutes of incubation time was significantly reduced for all tested dialysis fluids compared to the control medium RPMI. After 60 minutes of incubation the dehydrogenase activity dropped to 73 ± 11% for Glu/Bic pH 7.5 ($P < 0.01$, vs. RPMI), but was only 31 ± 8% for Glu/Lac pH 5.5 ($P < 0.001$, vs. RPMI). At all incubation times Glu/Bic and Amino/Bic fell nearest to the reference medium RPMI.

Effects of alternative dialysis solutions on apoptosis and necrosis

The rate of apoptotic cells after incubation with the test solutions was relatively uniform, and ranged between 20 and 28% with no significant differences between the fluids (Fig. 2). On the other hand, major differences could be registered for the rate of necrotic cells between the control medium (4.1 ± 1.1%) and the test solutions. The rate of necrotic cells after incubation with Glu/Bic pH 7.5 (7.1 ± 2.7%), Amino/Bic pH 7.5 (7.9 ± 3.1%) and Amino/PBS pH 7.5 (7.8 ± 2.9%) was similar to that of RPMI (NS). Glu/Lac pH 7.5 (10.4 ± 6.0) and Glu-poly/PBS (9.9 ± 2.2) were slightly elevated compared to RPMI ($P < 0.05$). A very high incidence of necrotic cells, however, was detected for Glu/Lac pH 5.5 (29.9 ± 4.0%; $P < 0.01$).

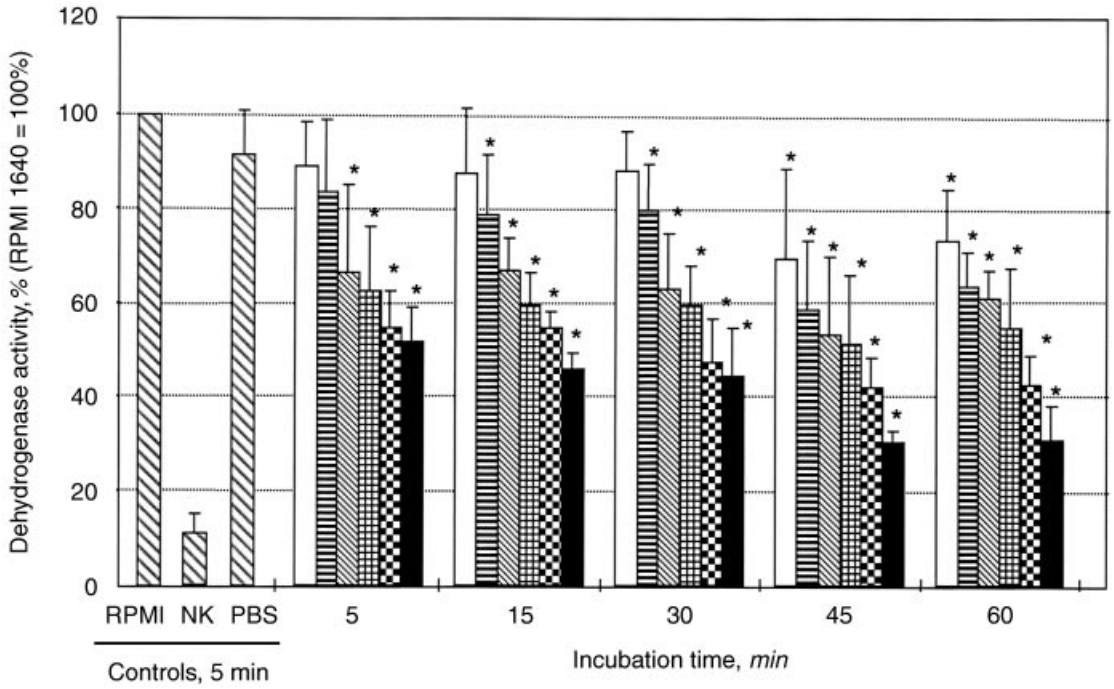

Fig. 1. Mitochondrial dehydrogenase activity measured in monocytes by the MTT assay. The change in enzyme activity on incubation with alternative dialysis solutions (5, 15, 30, 45, 60 min) was referred to RPMI 1640 as 100%. Abbreviation NK is the negative control, where the macrophages were killed by a preswitched freeze/thaw cycle and incubated with RPMI. Incubation with PBS buffer for five minutes was used as a positive control. All values are mean ± SD ($N = 4$). Statistical significance of the difference between control medium (RPMI 1640) and experimental fluids is given, $^*P < 0.05$. Symbols are: (□) Glu/Bic pH 7.5; (▤) Amino/Bic 7.5; (▧) Amino/PBS pH 7.5; ([squp]) Glu/Lac, pH 7.5; (◧) Glu-poly/PBS pH 7.4; (■) Glu/Lac pH 5.5.

Supravital staining with acridin orange using fluorescence microscopy confirmed this order of cell cytoxicity of the different fluids with the highest degree of cell necrosis found with Glu/Lac pH 5.5. Partial nuclear condensation but otherwise normal appearance was considered as an early sign of apoptosis. In subsequent phases of apoptosis nuclear condensation increased and the nucleus became yellowish. Characteristic bud-like cell surface protuberances could be observed. Necrotic cells showed a swollen or disrupted shape with a reddish appearance of the cytoplasma and the nucleus. A red fluorescence could also be observed in disintegrated cells as a result of late apoptosis, and thus could not always be distinguished from primary necrosis (Fig. 3). The rate of early apototic cells (with an intact cell membrane, corresponding to a negative PI staining in the FACS analysis) was evaluated. This ratio was nearly on the same level after 16 hours of incubation with the test fluids and ranged between 22% and 32%.

TUNEL positive cells had a bright nuclear fluorescence. The positive controls after the deliberate treatment with DNase I showed labeling of nearly all nuclei. The ratio of TUNEL positive cells in dialysate treated cultures varied between 24 and 35% (difference, NS; Fig. 3).

Influence of different dialysis solutions on the expression of housekeeping genes (β-actin, GAPDH)

The cellular β-actin mRNA expression after 15 minutes of incubation with the test fluids differed markedly (Figs. 4 and 5). While β-actin mRNA levels of Glu/Bic pH 7.5 (99 ± 2%), Amino/Bic pH 7.5 (90 ± 6%) and Amino/PBS pH 7.5 (90 ± 4%) were similar to the control medium (RPMI), we found a clear suppression for Glu/Lac pH 7.5 (68 ± 14%, $P < 0.05$), Glu-poly/PBS pH 7.4 (34 ± 15%, $P < 0.005$) and Glu/Lac pH 5.5 (11 ± 6%, $P < 0.001$). A parallel finding was observed for GAPDH mRNA expression after 15 minutes of incubation with the mentioned dialysates (data not shown). There was a clear correlation between mitochondrial dehydrogenase activity (measured by MTT-assay) and β-actin specific mRNA expression ($r = 0.877$, $P < 0.005$).

Effect of alternative dialysis solutions on IL-6 gene transcription

The ratio for IL-6/β-actin mRNA level was found to be > 0.6 for all tested fluids except Glu-poly/PBS pH 7.4, which showed a depressed IL-6/β-actin ratio of 0.2 ($P < 0.05$). The same pattern was noted when relating IL-6 to GAPDH mRNA expression (Fig. 5).

IL-6 and IL-8 release from LPS stimulated monocytes

IL-6 concentration in the cell supernatants after LPS stimulation was highest in the control media RPMI 1640 (1004 ± 354 pg/ml) and PBS (1154 ± 348 pg/ml). It was followed by Glu/Bic (1023 ± 278 pg/ml), Amino/PBS (1011 ± 226 pg/ml) and Amino/Bic (796 ± 296 pg/ml),

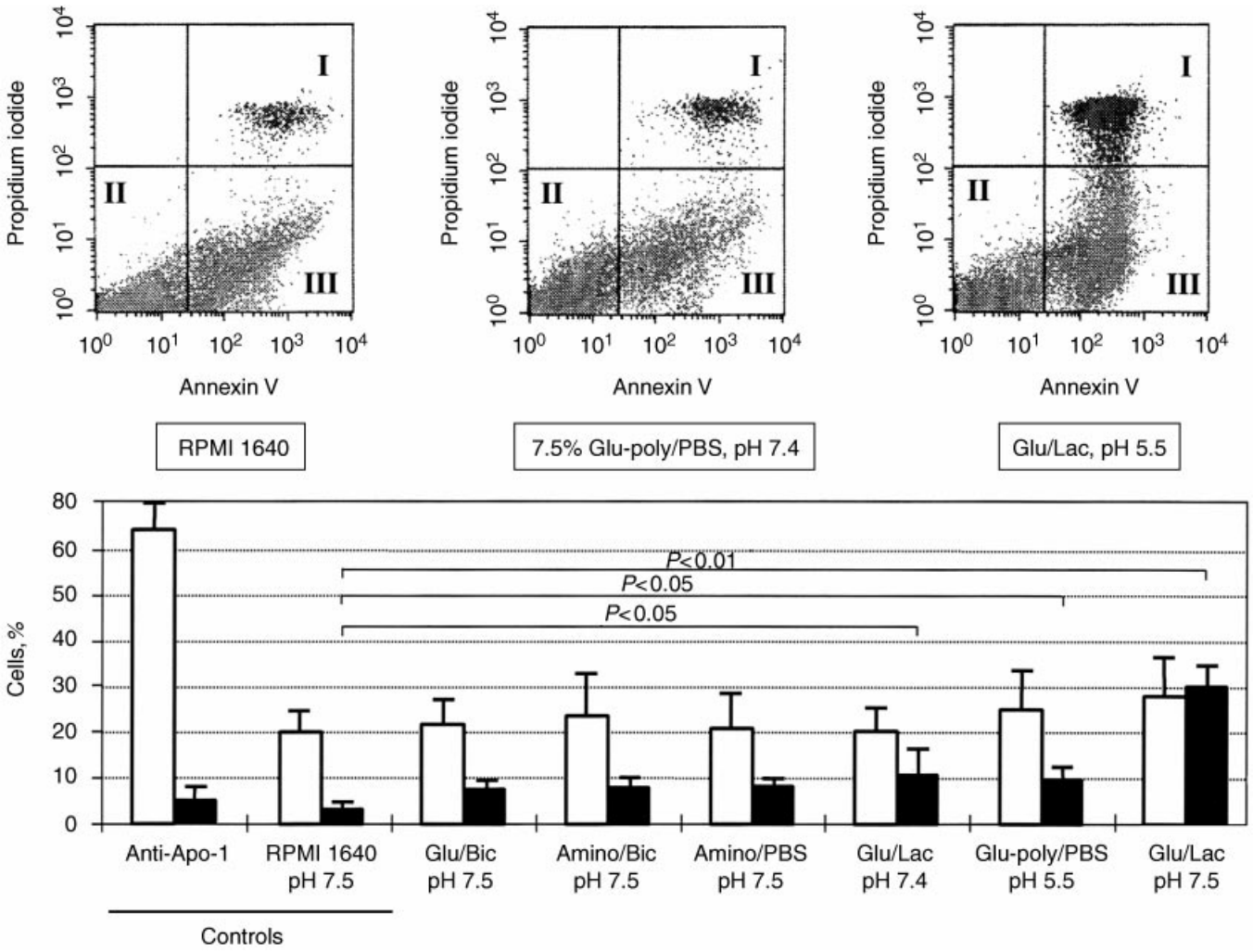

Fig. 2. Flow cytometric analysis of apoptotic and necrotic cells. Blood mononuclear cells were incubated for 15 minutes with the test dialysates. The cells (10^6/ml) were washed with bindung buffer and directly analyzed after addition of Annexin V^{FITC} and propidium iodide. Abbreviations in the boxes are: I, necrotic cells; II, intact cells; III, apoptotic cells. The bars indicate the relative distribution of apototic (□) and necrotic (■) cells measured by flow cytometry. A positive control for apoptosis was introduced by incubating MNCs with a monoclonal anti-Apo-1 antibody for two hours. Examples of orginal plots are demonstrated in the top half of this figure. All values are mean ± SD ($N = 5$).

which did not differ significantly. A low release of IL-6 was found for Glu/Lac pH 7.5 (267 ± 192 pg/ml), Glu/Lac pH 5.5 (46 ± 22 pg/ml) and 7.5% Glu-poly/PBS (32 ± 13 pg/ml). The lactate buffered glucose solution (Glu/Lac), even adjusted to a neutral pH of 7.5, was still inferior with regard to IL-6 release when compared to the bicarbonate buffered pendant Glu/Bic pH 7.5 (1023 pg/ml vs. 267 pg/ml, $P < 0.01$; Fig. 6).

The concentrations of IL-8 were about 10 times higher compared to IL-6 in the control media RPMI 1640 (9630 pg/ml ± 815) and PBS (8176 ± 681 pg/ml). The pattern of IL-8 release was similar to that of IL-6, but the differences between the fluids were somewhat less marked. Glu/Bic (9749 pg/ml ± 2038), Amino/Bic (9399 pg/ml ± 1560), Amino/PBS (9693 pg/ml ± 1301) and Glu/Lac pH 7.5 (9333 pg/ml ± 714) did not differ from the control medium. The 7.5% glucose polymer (3031 pg/ml ± 1586) and Glu/Lac pH 5.5 (2991 pg/ml ± 2143) showed a significantly reduced release (Fig. 6).

DISCUSSION

We studied the *in vitro* effects of alternative dialysate fluids that appear promising for clinical application. Peripheral blood monocytes were chosen for testing purposes in this study, because they are precursors of macrophages, available in high numbers, and have not been exposed to the potentially toxic dialysis fluids prior to study. A 5 to 60 minute lasting incubation of macrophages in undiluted dialysis fluid was tested beforehand, and appeared to be suited to simulate the *in vivo* situation with regard to pH influences or buffer systems. After 15 to 30 minutes, an equilibration of pH is reached in the peritoneal cavity of PD patients using standard Glu/Lac pH 5.5 solution [27]. On the other hand, osmolarity may be still increased after

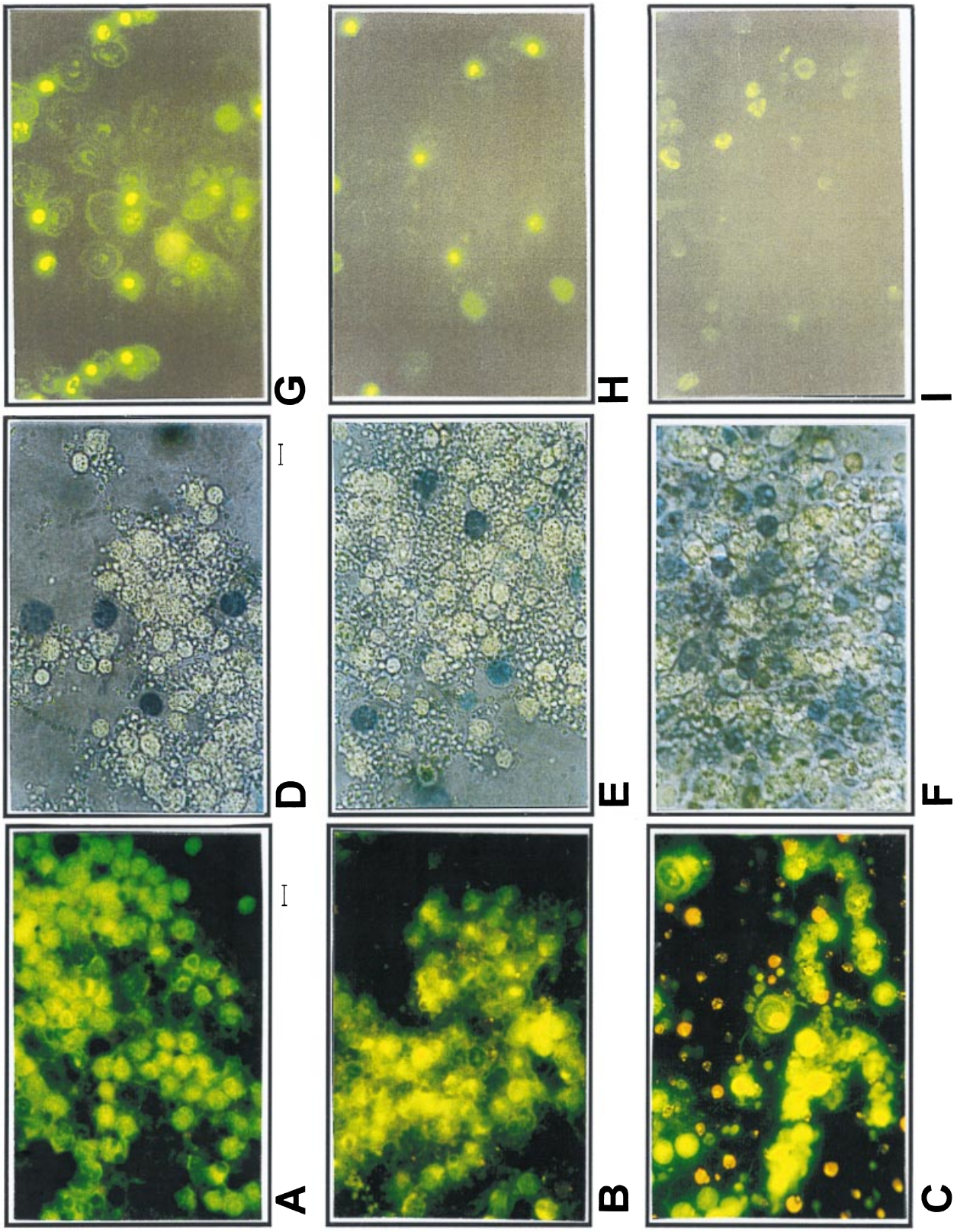

Fig. 3. Fluorescence microscopic pictures of unfixed macrophages stained with 10 μg/ml of AO, excitation at 490 nm (left side). Cells were incubated with RPMI control medium (*A*), Glu/Bic (*B*) or Glu/Lac (*C*). Viable cells displayed a green cytoplasmic fluorescence and some weaker nuclear fluorescence (double stranded DNA). Early apopotic cells showed nuclear fragmentation and condensation. In later phases of apoptosis nuclear condensation increased and the nucleus became yellowish. Cytoplasmic membrane blebbing increased (green buds). Necrotic cells showed a swollen cytoplasma with nuclear disintegration. Disrupted cells had a reddish appearance of the nucleus and the cytoplasma. These latter findings were predominantly observed with Glu/Lac (C). The cell membrane integrity was additionally tested by the trypan blue exclusion test (panel *D*, RPMI; *E*, Glu/Bic; *F*, Glu/Lac). Apoptosis detected by TUNEL-labeled nuclei of macrophages (right side). Pseudo-TUNEL-positive control cells were induced by DNase I treatment (*G*). Cultures were incubated with Glu/Bic (*H*) or conventional dialysis solution Glu/Lac (*I*). Bar = 20 μm

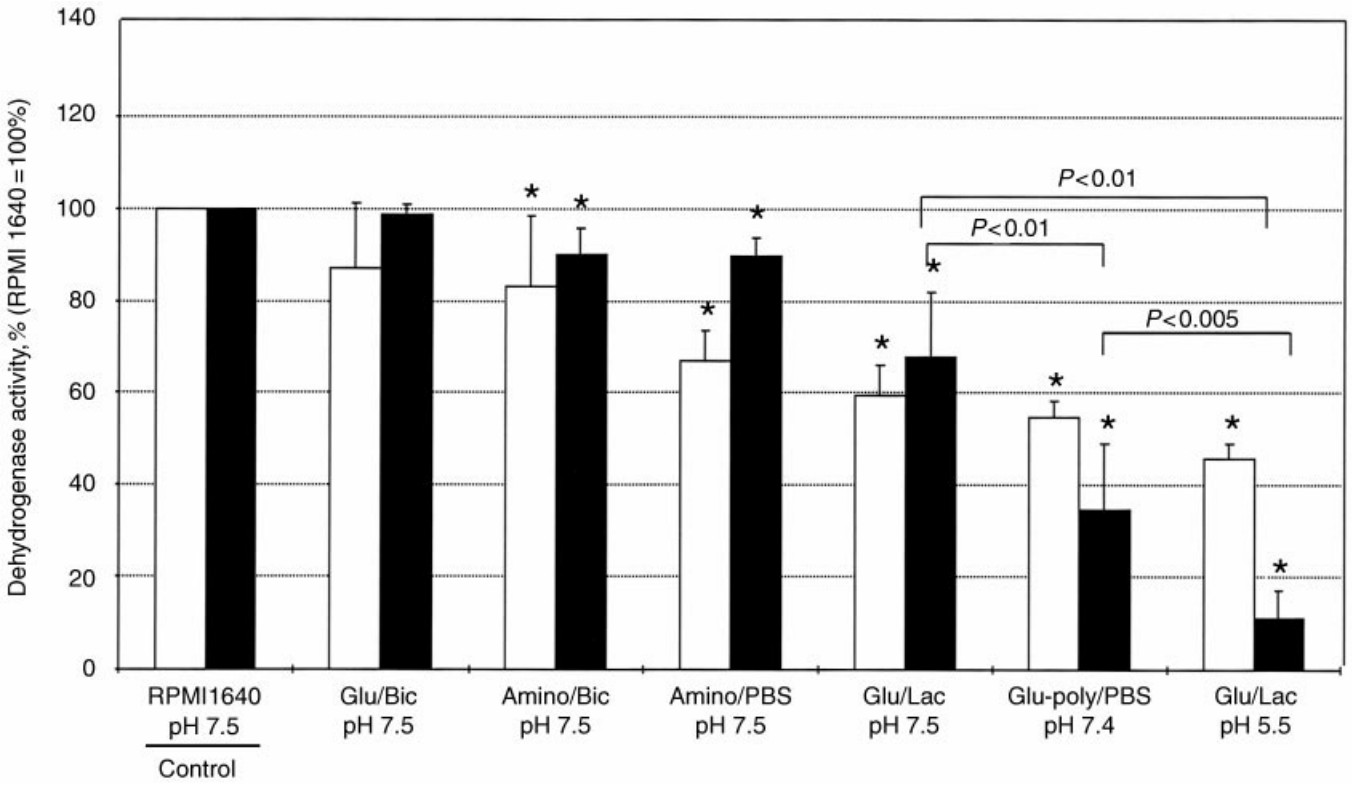

Fig. 4. Mitochondrial dehydrogenase activity (□; MTT assay) and β-actin specific mRNA expression (■; measured by RT-PCR) in blood monocytes incubated for 15 minutes with different dialysis solutions. Recovery period of 16 hours. β-actin expression was not different in non LPS stimulated cells or macrophages from peritonitis patients (see also Fig. 5). All values are mean ± SD ($N = 4$). Difference tested between control medium (RPMI 1640) and test fluids, $^*P < 0.05$. Correlation of β-actin/MTT, $r = 0.877$; $P < 0.005$.

a four hour dwell [27], and nutritional factors like hormones and proteins also accumulate slowly in the PD fluid [28]. For this purpose we used a coincubation design with undiluted dialysate exposure of the cells for 15 minutes (except for the MTT assay, where intervals between 5 to 60 min were evaluated) followed by a 1:1 dilution with RPMI and FCS for 16 hours. This procedure may simulate the *in vivo* equilibration more closely than a preincubation schedule.

On incubation times between five minutes to 60 minutes, the mitochondrial dehydrogenase activity progressively decreased with the test fluids compared to the control medium. However, according to the MTT test, the aerobic cell metabolism was predominantly compromised by lactate buffer in combination with a low pH. The clinical standard dialysate (1.36% Glu/Lac pH 5.5) resulted in a marked loss of mitochondrial dehydrogenase activity compared to RPMI at all incubation times. We observed a clear parallel between the dehydrogenase activity and the decrease of cellular β-actin mRNA expression after 15 minutes of incubation (Fig. 7). Principally, semiquantitative RT-PCR refers to a constant (unaltered) expression of a housekeeping gene. To verify the fact of a reduced β-actin mRNA transcription with some dialysates we also evaluated another housekeeping gene (GAPDH) in terms of mRNA levels, but the results were similar to that of β-actin, indicating severe cellular damage. The correlation between the mitochondrial dehydrogenase activity and the specific β-actin mRNA level was highly significant ($r = 0.877$, $P = 0.005$), also suggesting that a toxic effect of some test fluids resulted in reduced cellular mRNA transcripion or degradation. Necrotic cells are characterized by cell lysis [15, 16] and subsequently mRNA disintegration including housekeeping genes. To further clarify this situation we studied the rate of necrotic and apototic cells by FACS. After incubation with Glu/Lac pH 5.5, Glu/Lac pH 7.5 and 7.5% Glu-poly/PBS pH 7.4, we could show that the rate of necrotic cells was significantly increased compared to the control medium. The early state of apotosis as a form of programmed cell death (measured by the translocation of phophatidylserine to the cell surface), however, was not significantly different between the tested solutions. We quantitatively evaluated the apotosis/necrosis rate by FACS and could confirm our results by supravital acridine orange staining and the TUNEL assay. The rate of necrosis compared to the rate of apotosis registered, however, is also somewhat dependent on the incubation conditions. Cell lysis appearing as necrosis can be the final result of late apoptosis. Shorter incubation times with the test fluids indeed increased the ratio of apoptosis with a maximum

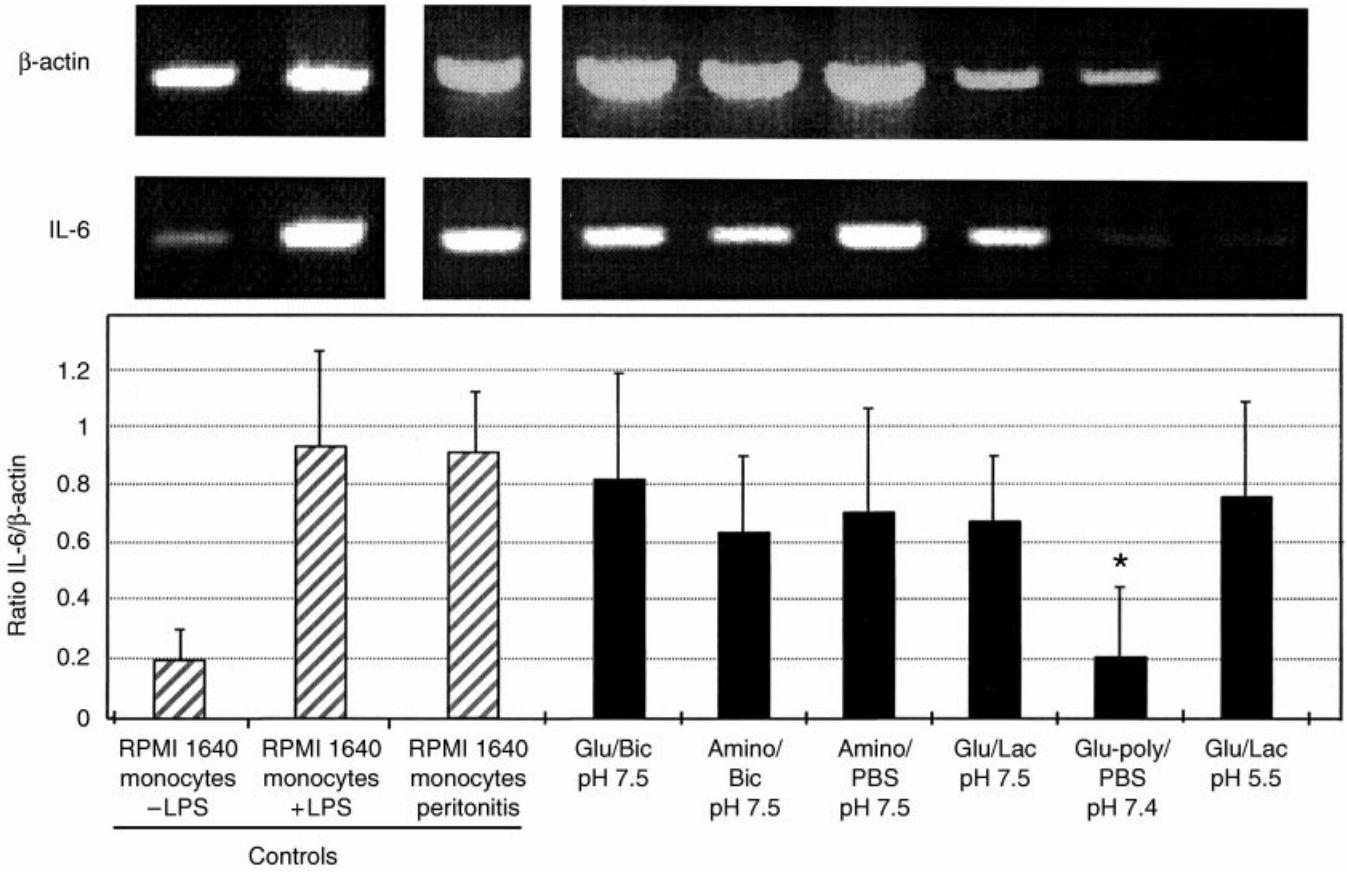

Fig. 5. Effect of alternative dialysates on β-actin and IL-6 mRNA expression in blood moncytes. β-Actin specific mRNA level (upper panel) and IL-6 mRNA (lower panel) were determined by RT-PCR. A total of 20 μl of each PCR reaction product was loaded onto a agarose gel containing ethidium bromide. Monocytes were activated with LPS (10 μg/ml). LPS and non-LPS stimulated blood moncytes incubated in RPMI served as controls. Activated macrophages from peritonitis patients were introduced as a control for the adequacy of LPS stimulation. All values are mean ± SD ($N = 6$). The IL-6/β-actin ratio was not different between all dialysates except for 7.5% glucose polymer pH 7.4. *$P < 0.05$, tested against control medium.

being noted after four to eight hours. Nevertheless, the high rates of cell necrosis occurring in rather early stages of incubation underline the high cytotoxity of some fluids.

One reason for the rapid loss of cell viability and the high incidence of necrosis may be a low intracellular pH. It has been shown that the combination of low pH and lactate as a buffer reduces cell viability and phagocytosis activity in leukocytes [5–7, 29]. This was attributed to a fast and sustained intracellular drop of pH and a subsequent inhibition of pH depending enzymes [6, 30, 31]. By this mechanism a prolonged disturbance of cellular host defence may be provoked (even after intraperitoneal pH equilibration) [32, 33].

As mentioned above, the β-actin and GAPDH housekeeping mRNA expression (measured by RT-PCR) was reduced with Glu/Lac pH 5.5. On a post-transcriptional level this may explain the low concentration of IL-6 measured in the cell supernatant for this dialysate. The IL-6/β-actin mRNA ratio, however, was not changed. We can assume, therefore, that transcriptional modulation is not responsible for the low secretion of IL-6 after LPS stimulation, but direct cytotoxicity of some dialysates that leads to cell damage and necrosis (Fig. 7). The expression of IL-6 mRNA induced by LPS and the release of IL-6/IL-8 in our test system was comparable to that found in activated peritoneal macrophages from patients with bacterial peritonitis, thereby indicating an appropriate *in vivo* stimulation.

Glucose polymer exerts a colloid osmotic effect giving sustained ultrafiltration due to a long lasting colloid osmotic gradient [34–36]. We found that 7.5% glucose polymer decreased cell viability, but also altered IL-6 mRNA expression in a specific manner. The test solution with 7.5% glucose polymer pH 7.4 showed a better cell viability compared to Glu/Lac pH 5.5 (with regard to MTT-assay and necrosis/apotosis assay), but was still inferior to Glu/Bic or Amino/Bic. Glucose polymer demonstrated an additional suppressive effect on IL-6 mRNA expression (IL-6/β-actin). This circumstance may explain the very low secretion of IL-6 into the cell supernatant upon LPS stimulation. Cell function may not only be compromised by hyperosmotic solutions as it is known from concentrated

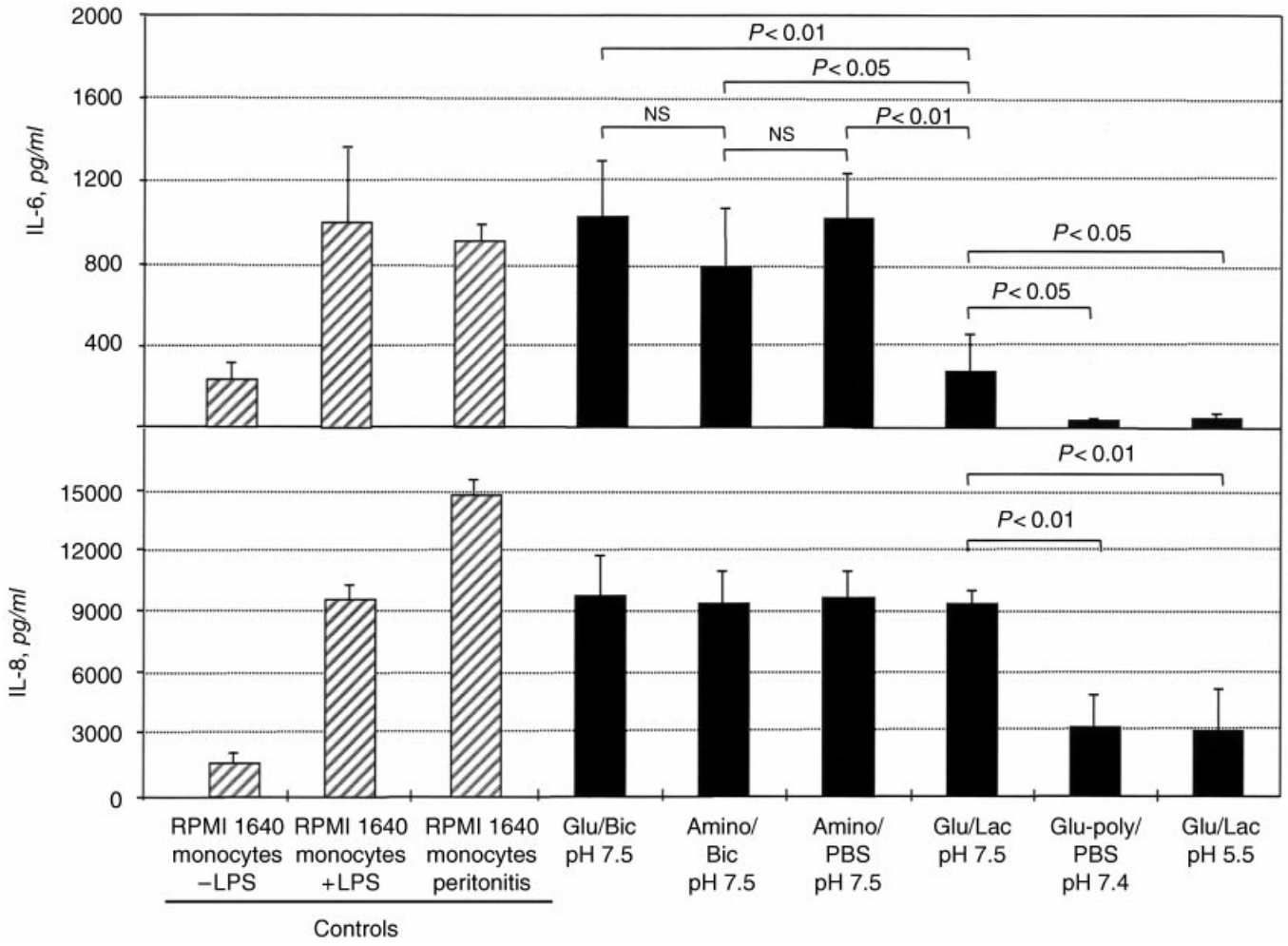

Fig. 6. Effect of alternative dialysates on LPS (10 μg/ml) stimulated IL-6 and IL-8 release from isolated blood monocytes (10×10^6). Incubation for 15 minutes with test dialysates, recovery period of 16 hours. Cytokine concentrations were measured in the cell supernatant after 16 hours by ELISA. LPS and non-LPS stimulated blood moncytes incubated in RPMI served as positive and negative controls. Activated macrophages from peritonitis patients were introduced as a control for the adequacy of LPS stimulation. All values are mean ± SD ($N = 6$).

glucose [6], but may also be influenced by a high colloid osmotic pressure. De Fijter et al showed that peritoneal macrophages of PD patients had better phagocytotic capacities and a higher oxidative metabolism after intraperitoneal exposure to the glucose polymer solution as compared with glucose monomer fluid [34]. A negative impact, on the other hand, was described by Liberek et al, who investigated mesothelial cells and neutrophile granulocytes [37]. Our results strengthen the impression that glucose polymer specifically influences the IL-6/IL-8 release of monocytes. The molecular weight of glucose polymer preparations and the type of branching (1-4, 1-6 glycosidic binding) could be aspects that influence biocompatibility patterns. The influence of the degradation products of the polymer should also be focused on in further studies.

Glu/Bic pH 7.5 and Amino/Bic pH 7.5 yielded the best results in terms of cell metabolism, viability and cytokine expression/release. Jörres et al also observed less suppression of IL-6 gene transcription and release after the exposure of mononuclear cells (MC) to bicarbonate buffered fluids with a physiological pH [7]. These data are in accordance to our results [38]. Dobos et al found that bicarbonate buffered fluids impaired much less neutrophile granulocyte function concerning phagocytosis, chemotaxis and change in cytoskeleton [39] compared to lactate solutions. Some benefit of bicarbonate over lactate may probably exist irrespective of pH [40], as our data comparing Glu/Lac and Glu/Bic show that both adjusted to a neutral pH. Chaimovitz et al and Zhou et al demonstrated that a high lactate concentration, even with a physiological pH value, resulted in a decreased intracellular pH [41, 42]. In our experiments the replacement of lactate by bicarbonate with glucose as well as with amino acids as osmotic agent resulted in a superior cytokine response and in an improvement of monocytic viability.

For clinical purposes, amino acids appear to be an

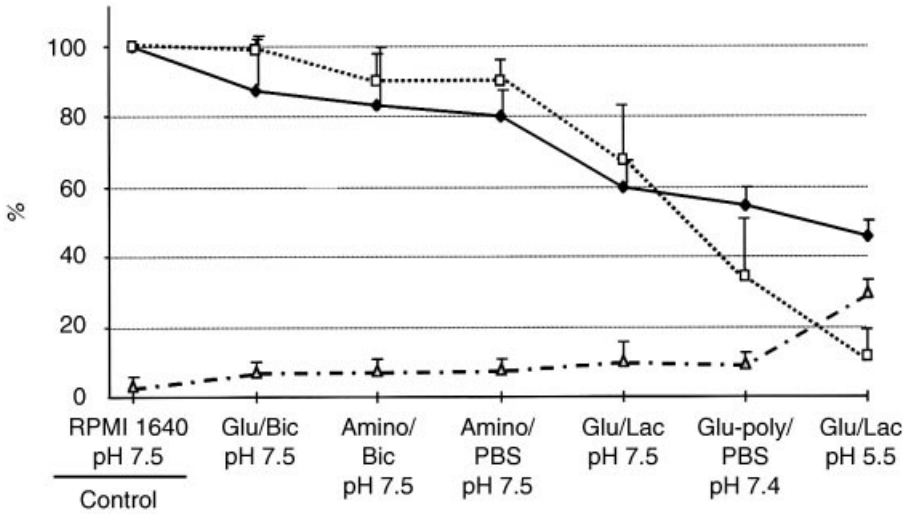

Fig. 7. Relationship between mitochondrial dehydrogenase activity (♦; MTT assay), β-actin mRNA expression (□; RT-PCR) and the rate of necrotic cells (△; propidium iodide staining, flow cytometry). Note the parallelism between metabolic cell activity and β-actin housekeeping gene expression and the inverse correlation to the extent of cell necrosis.

equivalent substitute for glucose with regards to dialysate biocompatiblity. A more widespread use of bicarbonate should be promoted, even if technical aspects of storage have to be improved further (such as, double chamber systems). Glucose polymer fluids appear to be a clinically attractive alternative in patients with low ultrafilration (highly permeable peritoneum) or in terms of reducing the glucose load. The *in vitro* findings with monocytes with regards to cell viability and a reduced cytokine release showed non-ideal conditions. Further *ex vivo* and *in vitro* research is ongoing in our center, with an emphasis on the effects of glucose polymer on isolated peritoneal macrophages after a long intraperitoneal dwell with this fluid. Biocompatibility research is also needed to examine the short-term and long-term effects of these new fluids on resident peritoneal cells (such as, mesothelial cells and fibroblasts) to determine if they are relevant for sustained peritoneal membrane integrity.

Reprint requests to Priv. Doz. Dr. Joerg Plum, Department of Nephrology and Rheumatology, Heinrich-Heine University Duesseldorf, Moorenstr. 5, 40225 Duesseldorf, Germany.
E-mail: plum@uni-duesseldorf.de

APPENDIX

Abbreviations used in this article are: Amino, amino acid dialysate; ATP, adenosine triphosphate; Bic, bicarbonate dialysate buffer; CAPD, continuous ambulatory peritoneal dialysis; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Glu, glucose dialysate; Glu-poly, glucose polymer (dextrin) dialysate; IL, interleukin; Lac, lactate dialysate buffer; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PD, peritoneal dialysis; PS, phosphatidylserine; RT-PCR, reverse transcription-polymerase chain reaction; TUNEL, TdT-mediated dUTP nick end labeling.

REFERENCES

1. Steinhauer HB, Schollmeyer P: Prostaglandin mediated loss of proteins during peritonitis in continuous ambulatory peritoneal dialysis. *Kidney Int* 29:584–590, 1986
2. Krediet RT, Zuyderhoudt FMJ, Bosschoeten EW, Arissz L: Alterations in the peritoneal transport of water and solutes during peritonitis in continuous ambulatory peritoneal dialysis patients. *Eur J Clin Invest* 17:43–52, 1987
3. Slingeneyer A, Canaud B, Mion C: Permanent loss of ultrafitration capacity of the peritoneum in long term peritoneal dialysis: An epidemiological study. *Nephron* 33:33–38, 1983
4. Cameron JS: Host defences in continuous ambulatory peritoneal dialysis and the genesis of peritonitis. *Pediatr Nephrol* 9:647–662, 1995
5. Plum J, Schoenicke G, Topley N, Grabensee B: Alternative osmotic agents and buffers in PD solutions–Influence on monocytic cytokine release and cell cytotoxicity. *Am J Kidney Dis* 30:413–422, 1997
6. Liberek T, Topley N, Jörres A, Coles GA, Gahl GM, Williams JD: Peritoneal dialysis fluid inhibition of phagocyte function: Effects of osmolality and glucose concentration. *J Am Soc Nephrol* 3:1508–1515, 1993
7. Jörres A, Gahl GM, Topley N, Neubauer A, Ludat K, Müller C, Passlick-Deetjen J: *In vitro* Biocompatibility of alternative CAPD fluids: Comparison of bicarbonate-buffered and glucose-polymer-based solutions. *Nephrol Dial Transplant* 9:785–790, 1994
8. Van Damme J, Van Beeumen J, Decock B, Van Snick J, Deley M, Biliau F: Separation and comparison of two monokines with indentification of leukocyte-derived HGF as interleukin-6. *J Immunol* 140:1534–1541, 1988
9. Zemel D, ten Berge RJM, Struijk DG, Bloemena E, Koomen GCM, Krediet RT: Interleukin-6 in CAPD patients without peritonitis: Relationship to the intrinsic permeability of the peritoneal membrane. *Clin Nephrol* 37:97–103, 1992
10. Shröder JM: The neutrophil-activating peptide (NAP-1/interleukin 8), a novel cytokine of the β-thromboglobulin supergene family, in *Natural Resistance to Infection* (vol 6) C. Jorg, ed. Stuttgart, Gustav Fischer Verlag, 1990, p 50
11. Matsushima K, Openheim JJ: Interleukin-8 and MCAF: Novel inflammatory cytokines inducible by IL-1 and TNF. *Cytokine* 1:2–13, 1989
12. Lindley I, Aschauer H, Seifert JM, Lam C, Brunowsky W, Kownatzki E, Thelen M, Peveri P, Dewalo B, Tscharner V: Synthesis and expression in *Escherichia coli* of the gene encoding monocyte-derived neutrophil-activating factor: Biological equivalence between natural and recombinant neutrophil-activating factor. *Proc Natl Acad Sci USA* 85:9199–203, 1988
13. Larsen CG, Anderson AO, Appela E, Oppenheim JJ, Matsushima K: The neutrophil-activating protein (NAP-1) is also chemotatic for T lymphocytes. *Science* 243:1464–1466, 1989
14. Leonard EJ, Skeel A, Yoshimura T, Noer K, Kutvirt S, van Epps D: Leucocyte specificity and binding of human neutrophil attractant/activator protein-1. *J Immmunol* 14:1323–1330, 1990

15. COHEN JJ: Programmed cell death in the immune system. *Adv Immunol* 50:55–85, 1991
16. ARENDS MJ, WYLLIE AH: Apoptosis. Mechanisms and roles in pathology. *Int Rev Exp Pathol* 32:223–254, 1991
17. WYLLIE AH, MORRIS RG, SMITH AL, DUNLOP D: Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular sysnthesis. *Am J Pathol* 142:67–77, 1984
18. KOOPMAN G, REUTELINGSPERGER CPM, KUIJTEN GAM, KEEHNEN RMJ, PALS ST, VAN OERS MHJ: Annexin V for flow cytometric detection of phosphatidylserine. Expression on B cells undergoing apoptosis. *Blood* 84:1415–1420, 1994
19. VERMES I, HAANEN C, STEFFENS-NAKKEN H, REUTELINGSPERGER C: A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immun Method* 184:39–51, 1995
20. BÖYUM A: Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 21(Suppl 97):77–89, 1968
21. BERRIDGE MV, TAN AS: Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys* 303:474–482, 1993
22. HANSEN MB, NIELSEN SE, BERG K: Reexamination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Method* 119:203–210, 1989
23. LOVE LD: Fluorescence microscopy of viable mast cells stained with different concentrations of acridine orange. *Histochemistry* 62:221–225, 1979
24. CHOMCZYNSKI P, SACCHI N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
25. NAKAJIMA-IJIMA S, HAMADA H, REDDY P, KAKUNAGA T: Molecular structure of human cytoplasmatic β-actin gene: Interspecies homolgy of sequence in the introns. *Proc Natl Acad Sci USA* 82:6133–6137, 1985
26. ZILBERSTEIN A, RUGGIERI R, KORN JH, REVEL M: Structure and expression of cDNA and genes for human interferon-β2 (IL-6), a distinct species inducible by growth-stimulatory cytokines. *EMBO J* 5:2529–2537, 1986
27. DUWE AK, VAS SI, WEATHERHEAD JW: Effects of the composition of peritoneal dialysis fluid on chemiluminescence, phagocytosis, and bactericidal activity in vitro. *Infect Immun* 33:130–135, 1981
28. JOERRES A, GAHL GM, FREI U: Peritoneal dialysis fluid biocompatibility: Does it really matter? *Kidney Int* 46(Suppl 48):S79–S86, 1994
29. TOPLEY N, MACKENZIE R, JÖRRES A, COLES GA, DAVIES M, WILLIAMS JD: Cytokine networks in continuous ambulatory peritoneal dialysis: Interactions of resident cells during inflammation in the peritoneal cavity. *Perit Dial Int* 13(Suppl 2):282–285, 1997
30. HOLMES CJ: Bocompatibility of peritoneal dialysis solutions. *Perit Dial Int* 13:88–94, 1993
31. ZHOU XJ, YU AM, ZHOU FQ, RYU J, ING TS, VAZIRI ND: Effects of an acidic, lactate-based peritoneal dialysis solution and its euhydric, bicarbonate-based counterpart on neutrophilic intracellular pH. *Int J Artif Organs* 16:816–819, 1993
32. TOPLEY N, ALOBAIDI HMM, DAVIES M, COLES GA, WILLIAMS JD, LLOYD D: The effect of dialysate on peritoneal phagocytose oxidative metabolism. *Kidney Int* 34:404–411, 1988
33. HAIN H, KESSEL M: Aspects of new solutions for peritoneal dialysis. *Nephrol Dial Transplant* 2:67–72, 1987
34. DE FIJTER CWH, HENRI H, VERBRUGH HA, OE LP, HEEZIUS E, DONKER AJ, VERHOEF J, GOKAL R: Biocompatibility of a glucose-polymer-containing peritoneal dialysis fluid. *Am J Kidney Dis* 21:411–418, 1993
35. MISTRY CD, GOKAL R, MALLICK NP: Glucose polymer as an osmotic agent in CAPD, in *Frontiers in Peritoneal Dialysis,* edited by MAHER JF, WINCHESTER JF, New York, Field Rich, 1986, pp 241–248
36. HO DAC PANNEKEET MM, SCHOUTEN N, LANGENDIJK MJ, HIRALALL JK, DE WAART DR, STRUIJK DG, KREDIET RT: Peritoneal transport characteristics with glucose polymer based dialysate. *Kidney Int* 50: 979–986, 1996
37. LIBEREK T, TOPLEY N, MISTRY CD, COLES GA, MORGEN T, QUIRK RA, WILLIAMS JD: Cell function and viability in glucose polymer peritoneal dialysis fluids. *Perit Dial Int* 13:104–111, 1993
38. PLUM J, FUBHÖLLER A, BUSCH G, SCHÖNICKE G, ERREN C, FIESLER C, PASSLICK-DEETJEN J, GRABENSEE B: In vivo and in vitro effects of an amino acid based and bicarbonate buffered peritoneal dialysis solutions with regard to peritoneal transport and cytokines/prostanoids dialysate concentrations. *Nephrol Dial Transplant* 12:1652–1660, 1997
39. DOBOS GJ, BÖHLER J, KUHLMANN J, ELSNER J, ANDRE M, PASSLICK-DEETJEN J, SCHOLLMEYER PJ: Biocarbonate-based dialysis solution preserves granulocyte functions. *Perit Dial Int* 14:366–379, 1994
40. THOMAS RC: Cell growth factors. Bicarbonate and pHi response. (news) *Nature* 16:337(6208):601, 1989
41. CHAIMOVITZ C, CONFORTI A, DOUVDEVANI A, YOM TG: The effect of commercial dialysis solutions on cytosolic pH of peritoneal macrophages. *Perit Dial Int* 12(Suppl 1):106, 1992
42. ZHOU XJ, YU AW, ZHOU FQ, WONG FK, ING TS, VAZIRI ND: Neutrophil intracellular pH after exposure of neutrophils to a euhydric, lactate-based peritoneal dialysis solution and its euhydric, bicarbonate-based counterpart. *Int J Artif Organs* 18:69–72, 1995